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(57) Abstract

The *Arabidopsis RPP5* gene has been cloned and its sequence provided, along with the encoded amino acid sequence. DNA encoding the polypeptide, and alleles, mutants and derivatives thereof, may be introduced into plant cells and the encoded polypeptide expressed, conferring pathogen resistance on plants comprising such cells and descendants thereof. The *RPP5* sequence comprises leucine rich repeats and the presence of such repeats enables identification of other plant pathogen resistance genes. Homologies between *RPP5* and other pathogen resistance genes reveal motifs useful in identification of other pathogen resistance genes.

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PLANT PATHOGEN RESISTANCE GENES AND USES THEREOF

The present invention relates to pathogen resistance in plants and more particularly to the identification and use of pathogen resistance genes. It is based on cloning of the *Arabidopsis RPP5* gene.

Plants are constantly challenged by potentially pathogenic microorganisms. Crop plants are particularly vulnerable, because they are usually grown as genetically uniform monocultures; when disease strikes, losses can be severe. However, most plants are resistant to most plant pathogens. To defend themselves, plants have evolved an array of both preexisting and inducible defences. Pathogens must specialize to circumvent the defence mechanisms of the host, especially those biotrophic pathogens that derive their nutrition from an intimate association with living plant cells. If the pathogen can cause disease, the interaction is said to be compatible, but if the plant is resistant, the interaction is said to he incompatible. Race specific resistance is strongly correlated with the hypersensitive response (HR), an induced response by which (it is hypothesized) the plant deprives the pathogen of living host cells by localized cell death at sites of attempted pathogen ingress.

It has long been known that HR-associated disease resistance is often (though not exclusively) specified by dominant genes (*R* genes). Flor showed that when

pathogens mutate to overcome such *R* genes, these mutations are recessive. Flor concluded that for R genes to function, there must also be corresponding genes in the pathogen, denoted avirulence genes (Avr genes). To become virulent, pathogens must thus stop making a product that activates *R* gene-dependent defence mechanisms (Flor, 1971). A broadly accepted working hypothesis, often termed the elicitor/receptor model, is that *R* genes encode products that enable plants to detect the presence of pathogens, provided said pathogens carry the corresponding *Avr* gene (Gabriel and Rolfe, 1990). This recognition is then transduced into the activation of a defence response.

Some interactions exhibit different genetic properties. *Helminthosporium carbonum* races that express a toxin (Hc toxin) infect maize lines that lack the Hml resistance gene. Mutations to loss of Hc toxin expression are recessive, and correlated with loss of virulence, in contrast to gene-for-gene interactions in which mutations to virulence are recessive. A major accomplishment was reported in 1992, with the isolation by tagging of the *Hml* gene (Johal and Briggs, 1992). Plausible arguments have been made for how gene-for-gene interactions could evolve from toxin-dependent virulence. For example, plant genes whose products were the target of the toxin might mutate to confer even greater sensitivity

to the toxin, leading to HR, and the conversion of a sensitivity gene to a resistance gene. However, this does not seem to be the mode of action of *Hm1*, whose gene product inactivates Hc toxin.

Pathogen avirulence genes are still poorly understood. Several bacterial *Avr* genes encode hydrophilic proteins with no homology to other classes of protein, while others carry repeating units whose number can he modified to change the range of plants on which they exhibit avirulence (Keen, 1992; Long and Staskawicz, 1993). Additional bacterial genes (*hrp* genes) are required for bacterial *Avr* genes to induce HR, and also for pathogenicity (Keen, 1992; Long and Staskawicz, 1993). It is not clear why pathogens make products that enable the plant to detect them. It is widely believed that certain easily discarded *Avr* genes contribute to but are not required for pathogenicity, whereas other *Avr* genes are less dispensable (Keen, 1992; Long, et al, 1993). The characterization of two fungal avirulence genes has also been reported. The *Avr9* gene of *Cladosporium fulvum*, which confers avirulence on *C. fulvum* races that attempt to attack tomato varieties that carry the *Cf-9* gene, encodes a secreted cysteine-rich peptide with a final processed size of 28 amino acids but its role in compatible interactions is not clear (De Wit, 1992). The *Avr4* gene of *C. fulvum* encodes a secreted peptide that is processed to a final size of amino

acids 106 (Joosten *et al*, 1994)

The technology for gene isolation based primarily on genetic criteria has improved dramatically in recent years, and many workers are currently attempting to clone a variety of *R* genes.

The map based cloning of the tomato *Pto* gene that confers "gene-for-gene" resistance to the bacterial speck pathogen *Pseudomonas syringae pv* tomato (Pst) has been reported (Martin *et al*, 1993). A YAC (yeast artificial chromosome) clone was identified that carried restriction fragment length polymorphism (RFLP) markers that were very tightly linked to the gene. This YAC was used to isolate homologous cDNA clones. Two of these cDNAs were fused to a strong promoter, and after transformation of a disease sensitive tomato variety, one of these gene fusions was shown to confer resistance to Pst strains that carry the corresponding avirulence gene, *AvrPto*. These two cDNAs show homology to each other. Indeed, the *Pto* cDNA probe reveals a small gene family of at least six members, 5 of which can be found on the YAC from which *Pto* was isolated, and which thus comprise exactly the kind of local multigene family inferred from genetic analysis of other *R* gene loci.

The *Pto* gene cDNA sequence is puzzling for proponents of the simple elicitor/receptor model. It reveals unambiguous homology to serine/threonine kinases, consistent with a role in signal

transduction. Intriguingly, there is strong homology to the kinases associated with self incompatibility in Brassicas, which carry out an analogous role, in that they are required to prevent the growth of genotypically defined incompatible pollen tubes. However, in contrast to the Brassica SRK kinase (Stein *et al*, 1991), the *Pto* gene appears to code for little more than the kinase catalytic domain and a potential N-terminal myristoylation site that could promote association with membranes. It would be surprising if such a gene product could act alone to accomplish the specific recognition required to initiate the defence response only when the *AvrPto* gene is detected in invading microorganisms. The race-specific elicitor molecule made by Pst strains that carry *AvrPto* is still unknown and needs to he characterized before possible recognition of this molecule by the Pto gene product can be investigated.

Since the isolation of the *Pto* gene a number of other resistance genes have been isolated. The isolation of the tobacco mosaic resistance gene *N* from tobacco was reported by Whitham *et al* (1994). The isolation of the flax rust resistance gene *L6* from flax was reported by Lawrence *et al* (1995). The isolation of two *Arabidopsis thaliana* genes for resistance to *Pseudomonas syringae* has been reported. The isolation of *RPS2* was reported by Bent *et al* (1994) and by Mindrinos *et al* (1994) and the isolation

of *RPM1* was reported by Grant et *al* (1995). These genes probably encode cytoplasmic proteins that carry a nucleotide binding site (NBS) and a leucine-rich repeat (LRR). The ligands with which they interact are uncharacterised and it is not known what other plant proteins they interact with to accomplish the defence response. Our own laboratory has reported the isolation of the tomato *Cf-9* gene which confers resistance against the fungus *Cladosporium fulvum*. This is disclosed in WO95/18230 and has been reported in Jones *et al* (1994). We have also cloned the tomato *Cf-2* gene, which confers resistance against *Cladosporium fulvum;* this is disclosed in an International patent application filed by us on 1 April 1996 claiming priority from GB 9506658.5 filed 31 March 1995 and has been reported in Dixon *et al.* (1996). Its structure resembles the *Cf-9* gene in that the DNA sequence predicts a protein which is predominantly extracellular, with many leucine-rich repeats and which carries a C-terminal putative membrane anchor. The *Xa21* gene of rice has also been cloned recently (Song *et al.*, 1995). The predicted protein product of this gene exhibits an N-terminal, presumably extracellular, domain composed principally of leucine rich repeats similar to those of *Cf-9* and *Cf-2*, a predicted transmembrane domain, and a presumably cytoplasmic domain with strong similarities to serine-threonine protein kinases, particularly

that encoded by *Pto*.

The subject-matter of the present invention relates to "pathogen resistance genes" or "disease resistance genes" and uses thereof. A pathogen resistance gene (R) enables a plant to detect the presence of a pathogen expressing a corresponding avirulence gene (Avr). When the pathogen is detected, a defence response such as the hypersensitive response (HR) is activated. By such means a plant may deprive the pathogen of living cells by localised cell death at sites of attempted pathogen ingress. Other genes, including the PGIP gene of WO93/11241 (for example), are induced in the plant defence response resulting from detection of a pathogen by an R gene.

A pathogen resistance gene may be envisaged as encoding a receptor to a pathogen-derived and Avr dependent molecule. In this way it may be likened to the RADAR of a plant for detection of a pathogen. Genes involved in the defence the plant mounts to the pathogen once detected are not pathogen resistance genes. Expression of a pathogen resistance gene in a plant causes activation of a defence response in the plant. This may be upon contact of the plant with a pathogen or a corresponding elicitor molecule, though the possibility of causing activation by over-expression of the resistance gene in the absence of elicitor has been reported. The defence response may be activated locally, e.g. at a site of contact of the

plant with pathogen or elicitor molecule, or systemically. Activation of a defence response in a plant expressing a pathogen resistance gene may be caused upon contact of the plant with an appropriate, corresponding elicitor molecule. The elicitor may be contained in an extract of a pathogen such as *Peronospora parasitica*, or may be wholly or partially purified and may be wholly or partially synthetic. An elicitor molecule may be said to "correspond" if it is a suitable ligand for the R gene product to elicit activation of a defence response.

We have now isolated the *Arabidopsis RPP5* gene which confers resistance against the downy mildew fungus (*Peronospora parasitica*). We have sequenced the DNA and deduced the most likely amino acid sequence from this gene. The DNA sequence of the *Arabidopsis RPP5* genomic gene is shown in Figure 1 (SEQ ID NO. 1) and the deduced amino acid sequence is shown in Figure 2 (SEQ ID NO. 2). The prediction of the Amino acid sequence is based on the identification of introns by reverse transcriptase polymerase chain reaction using primers designed to the determined genomic sequence. The part of the DNA sequence that is presumed to be spliced into exons and encoding the *RPP5* polypeptide is shown in capital letters in Figure 1. Figure 4 (SEQ ID NO 5) shows a contiguous nucleotide sequence coding for the amino acid sequence of Figure 2, made by joining together the exons of the

sequence of Figure 1.

As described in more detail below, the *Arabidopsis RPP5* gene was isolated by map-based cloning. In this technique the locus that confers resistance is mapped at high resolution relative to restriction fragment length polymorphism (RFLP) markers that are linked to the resistance gene. We identified a marker that appeared to be absolutely linked to the resistance gene and used probes corresponding to this marker to isolate binary vector cosmid clones from a library made with DNA of an *Arabidopsis* landrace *Landsberg erecta* that carried the *RPP5* gene. A binary vector cosmid clone designated 29L17, on transformation into disease sensitive *Arabidopsis*, conferred disease resistance. DNA sequence analysis of the cloned DNA identified a gene with leucine-rich repeats. A subclone of 29L17, designated pRPP5-1, containing 6304 bp of DNA including 1298 bp 5' to the probable initiation codon (Figure 1) and 458 bp 3' to the probable termination codon was constructed in a binary vector. The subclone was used to transform *Arabidopsis* ecotype Columbia and shown to confer disease resistance. Analysis of a fast neutron induced mutation of *Landsberg* that had become disease sensitive revealed rearrangement of the DNA structure of this gene. Taken together these data provide the necessary evidence that the sequences as shown in Figures 1 and 2 correspond to the *RPP5* gene.

According to one aspect, the present invention provides a nucleic acid isolate encoding a pathogen resistance gene, the gene being characterized in that it encodes the amino acid sequence shown in SEQ ID NO 2, or a fragment thereof, or an amino acid sequence showing a significant degree of homology thereto. N and L6 may be excluded.

For instance, embodiments of nucleic acid according to the invention, e.g. encoding a polypeptide comprising an amino acid sequence that is a mutant, derivative, allele or variant of the sequence shown in Figure 2 (as discussed further herein), may be distinguished from other pathogen resistance genes such as N, L6 by optionally having any one or more of the following features:

the encoded polypeptide has less than 30% homology with the amino acid sequence of the tobacco N protein, shown in Figure 3 and less than 25% homology with the amino acid sequence of the flax L6 protein, shown in Figure 3;

its expression does not activate said defence response upon contact of the plant with a molecule that is an elicitor of the tobacco N protein;

its expression does not activate said defence response upon contact of the plant with a molecule that is an elicitor of the flax L6 protein;

its expression does not when in a tobacco plant activate said defence response upon contact of the

tobacco plant with Tobacco Mosaic Virus;

its expression does not when in a flax plant activate said defence response upon contact of the flax plant with *Melampsora lini*;

its expression does not activate said defence response upon contact of the plant with a molecule that is an elicitor of the *Arabidopsis* RPS2 protein;

its expression does not activate said defence response upon contact of the plant with a molecule that is an elicitor of the *Arabidopsis* RPM1 protein;

its expression does not when in *Arabidopsis thaliana* activate said defence response upon contact of the plant with *Pseudomonas syringae*;

the encoded polypeptide shows less than 20% homology with the amino acid sequence of the tomato Cf-9 protein and less than 20% homology with the amino acid sequence of a tomato Cf-2 protein;

its expression does not activate said defence response upon contact of the plant with a molecule that is an elicitor of the tomato Cf-9 protein nor with a molecule that is an elicitor of the tomato Cf-2 protein;

its expression does not when in a tomato plant activate said defence response upon contact of the tomato plant with *Cladosporium fulvum* expressing an Avr2 molecule nor *Cladosporium fulvum* expressing an Avr9 molecule;

the encoded polypeptide comprises a putative

nucleotide binding site;

the encoded polypeptide is a cytoplasmic protein;

the encoded polypeptide comprises a region having homology to the cytoplasmic domain of the *Drosophila* Toll protein.

Another way of distinguishing nucleic acid according to the present invention from other pathogen resistance genes such as N and L6 may be for the encoded polypeptide to comprise an N-terminal domain that has greater than 60% homology with the amino acid sequence of the N-terminal domain of RPP5 shown in Figure 2 (encoded by exon 1 of Figure 1), and/or comprise a nucleotide binding site domain that has greater than 40% homology with the amino acid sequence of the domain of RPP5 shown in Figure 2 encoded by exon 2 of Figure 1, and/or comprise a domain that has greater than 30% homology with the amino acid sequence of the domain of RPP5 shown in Figure 2 encoded by exon 3 of Figure 1, and/or comprise a domain that has greater than 30% homology with the amino acid sequence of the leucine-rich repeat (LRR) domain of RPP5 shown in Figure 2 encoded by exons 4, 5 and 6 of Figure 1.

Table 2 shows % amino acid identity between putative domains of RPP5 and N, and RPP5 and L6, as encoded by exons of the genomic sequences.

The nucleic acid may comprise a sequence of nucleotides encoding an amino acid sequence showing at least about 60% homology, preferably at least about

70% homology, at least about 80% homology, or more preferably at least about 90% or greater homology to the amino acid sequence shown in SEQ ID NO 2. Generally, "% amino acid homology" is used to refer to % amino acid identity. High homology may be indicated by ability of complementary nucleic acid to hybridise under appropriate conditions, for instance conditions stringent enough to exclude hybridisation to sequences not encoding a pathogen resistance gene. Thus, the words allele, derivative or mutant may in context be used in respect of any sequence of nucleotides capable of hybridising with any of the nucleotide sequences encoding a polypeptide comprising the relevant sequence of amino acids.

Most preferably the nucleic acid encodes the amino acid sequence shown in SEQ ID No 2 in which case the nucleic acid may comprise DNA with an encoding sequence shown in SEQ ID NO 1 or sufficient part to encode the desired polypeptide (eg from the initiating methionine codon to the first in frame downstream stop codon of the mRNA). In one embodiment, DNA comprises a sequence of nucleotides which are the nucleotides 1966 to 6511 of SEQ ID NO 1, or a mutant, derivative or allele thereof, for instance lacking introns. Figure 4 provides a contiguous sequence encoding the amino acid sequence of Figure 2.

A further aspect of the invention provides a nucleic acid isolate encoding a pathogen resistance

gene, or a fragment thereof, obtainable by screening a nucleic acid library with a probe comprising nucleotides 1966 to 6511 of SEQ ID NO 1, nucleotides complementary thereto, or a fragment, derivative, mutant or allele thereof, and isolating nucleic acid which encodes a polypeptide able to confer pathogen resistance to a plant. Suitable techniques are well known in the art. Thus, the present invention also provides a method of identifying and/or isolating nucleic acid encoding a pathogen resistance gene comprising probing candidate (or "target") nucleic acid with nucleic acid which has a sequence of nucleotides which encodes the amino acid sequence shown in Figure 2, which is complementary to an encoding sequence or which encodes a fragment of either an encoding sequence or a sequence complementary to an encoding sequence. The candidate nucleic acid (which may be, for instance, cDNA or genomic DNA) may be derived from any cell or organism which may contain or is suspected of containing nucleic acid encoding a pathogen resistance gene. A preferred nucleotide sequence appears in Figure 1. Sequences complementary to the sequence shown, and fragments thereof, may be used.

Preferred conditions for probing are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further. It is

well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain.

Nucleic acid according to the present invention may encode the amino acid sequence shown in SEQ ID NO 2 or a mutant, derivative or allele of the sequence provided. Preferred mutants, derivatives and alleles are those which retain a functional characteristic of the protein encoded by the wild-type gene, especially the ability to confer pathogen resistance. Changes to a sequence, to produce a mutant or derivative, may be by one or more of insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the insertion, deletion or substitution of one or more amino acids. Of course, changes to the nucleic acid which make no difference to the encoded amino acid sequence are included.

The nucleic acid may be DNA or RNA and may be synthetic, eg with optimised codon usage for expression in a host organism of choice. Nucleic acid molecules and vectors according to the present invention may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of nucleic acid or genes of the species of interest or origin other than the sequence encoding a polypeptide with the required function. Nucleic acid according to the present invention may comprise cDNA, RNA, genomic

DNA and may be wholly or partially synthetic. The term "isolate" encompasses all these possibilities.

Also provided by an aspect of the present invention is nucleic acid comprising a sequence of nucleotides complementary to a nucleotide sequence hybridisable with any encoding sequence provided herein. Another way of looking at this would be for nucleic acid according to this aspect to be hybridisable with a nucleotide sequence complementary to any encoding sequence provided herein. Of course, DNA is generally double-stranded and blotting techniques such as Southern hybridisation are often performed following separation of the strands without a distinction being drawn between which of the strands is hybridising. Preferably the hybridisable nucleic acid or its complement encode a polypeptide able to confer pathogen resistance on a host, i.e. includes a pathogen resistance gene. Preferred conditions for hybridisation are familiar to those skilled in the art, but are generally stringent enough for there to be positive hybridisation between the sequences of interest to the exclusion of other sequences, i.e. sequences not encoding polypeptides able to confer pathogen resistance on a host.

The nucleic acid may be in the form of a recombinant vector, for example a phage or cosmid vector. The nucleic acid may be under the control of an appropriate promoter and regulatory elements for

expression in a host cell, for example a plant cell. In the case of genomic DNA, this may contain its own promoter and regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter and regulatory elements for expression in the host cell.

Those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al*, 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The

nucleic acid to be inserted may be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material may or may not occur according to different embodiments of the invention. Finally, as far as plants are concerned the target cell type must be such that cells can be regenerated into whole plants.

Plants transformed with a DNA segment containing pre-sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966), electroporation (EP 290395, WO 8706614) or other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611). Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Although Agrobacterium has been reported to be able to transform foreign DNA into some monocotyledonous species (WO 92/14828),

microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg. bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention.

The *RPP5* gene, modified versions thereof and related genes encoding a protein showing a significant degree of homology to the protein product of the *RPP5* gene, alleles, mutants and derivatives thereof, may be used to confer pathogen resistance, e.g. to downy mildews, in plants. For this purpose nucleic acid as described above may be used for the production of a transgenic plant. Such a plant may possess pathogen resistance conferred by the *RPP5* gene.

The invention thus further encompasses a host

cell transformed with a vector as disclosed, especially a plant or a microbial cell. Thus, a host cell, such as a plant cell, comprising nucleic acid according to the present invention is provided. Within the cell, the nucleic acid may be incorporated within the chromosome.

A vector comprising nucleic acid according to the present invention need not include a promoter, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

Also according to the invention there is provided a plant cell having incorporated into its genome a sequence of nucleotides as provided by the present invention, under operative control of a promoter for control of expression of the encoded polypeptide. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector comprising the sequence of nucleotides into a plant cell. Such introduction may be followed by recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome. The polypeptide encoded by the introduced nucleic acid may then be expressed.

A plant which comprises a plant cell according to the invention is also provided, along with any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as

cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on.

The invention further provides a method comprising expression from nucleic acid encoding the amino acid sequence SEQ ID NO 2, or a mutant, allele or derivative thereof, or a significantly homologous amino acid sequence, within cells of a plant (thereby producing the encoded polypeptide), following an earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof. Such a method may confer pathogen resistance on the plant.

A gene stably incorporated into the genome of a plant is passed from generation to generation to descendants of the plant, cells of which descendants may express the encoded polypeptide and so may have enhanced pathogen resistance. Pathogen resistance may be determined by assessing compatibility of a pathogen such as *Peronospora parasitica* or *Bremia lactucae*.

Sequencing of the *RPP5* gene has shown that like the *Cf-9* gene and the *Cf-2* gene it includes DNA sequence encoding leucine-rich repeat (LRR) regions and homology searching has revealed strong homologies to other genes containing LRRs. As discussed in WO95/18230, and further validated in this discovery, the presence of LRRs may be characteristic of many pathogen resistance genes and the presence of LRRs can

thus be used in a method of identifying further pathogen resistance genes.

Furthermore, there are some striking homologies between *RPP5* and the tobacco mosaic virus resistance gene *N* and the flax rust resistance gene *L6*. (Figure 3). (As can be derived from Figure 3, the overall homology between RPP5 and N is 33% amino acid identity, while the figure for RPP5 and L6 is 27%.) These homologies may also be used to identify further resistance genes, for example using oligonucleotides (e.g. a degenerate pool) designed on the basis of sequence conservation, preferably conservation of amino acid sequence. In particular, primers may be designed that amplify DNA between the regions of the gene that encode the amino acid sequence F Y D V D P (SEQ ID NO 6) of *RPP5* and *N* and where in *L6* it encodes F Y M V D P (SEQ ID NO 7), and the region I A C F F (SEQ ID NO 8) of *RPP5*, where the sequence is identical in *L6* and in *N* is I A C F L (SEQ ID NO 9).

According to a further aspect, the present invention provides a method of identifying a plant pathogen resistance gene comprising use of an oligonucleotide(s) which comprise(s) a sequence or sequences that are conserved between pathogen resistance genes such as *RPP5*, N and L6 to search for new resistance genes. Thus, a method of obtaining nucleic acid comprising a pathogen resistance gene (encoding a polypeptide able to confer pathogen

resistance) is provided, comprising hybridisation of an oligonucleotide (details of which are discussed herein) or a nucleic acid molecule comprising such an oligonucleotide to target/candidate nucleic acid. Target or candidate nucleic acid may, for example, comprise a genomic or cDNA library obtainable from an organism known to encode a pathogen resistance gene. Successful hybridisation may be identified and target/candidate nucleic acid isolated for further investigation and/or use.

Hybridisation may involve probing nucleic acid and identifying positive hybridisation under suitably stringent conditions (in accordance with known techniques) and/or use of oligonucleotides as primers in a method of nucleic acid amplification, such as PCR. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further. It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain.

As an alternative to probing, though still employing nucleic acid hybridisation, oligonucleotides designed to amplify DNA sequences may be used in PCR reactions or other methods involving amplification of nucleic acid, using routine procedures. See for instance "PCR protocols; A Guide to Methods and

Applications", Eds. Innis *et al*, 1990, Academic Press, New York.

Preferred amino acid sequences suitable for use in the design of probes or PCR primers are sequences conserved (completely, substantially or partly) between at least two polypeptides able to confer pathogen resistance such as those encoded by *RPP5* and *N* and/or *L6*.

On the basis of amino acid sequence information oligonucleotide probes or primers may be designed, taking into account the degeneracy of the genetic code, and, where appropriate, codon usage of the organism from the candidate nucleic acid is derived. Preferred nucleotide sequences may include those comprising or having a sequence encoding amino acids (i) F Y D V D P (SEQ ID NO 6); (ii) I A C F F (SEQ ID NO 8) or a sequence complementary to these encoding sequences. Suitable fragments of these may be employed. For example, the oligonucleotide TTC/T TAC/T GAC/T GTX GAT/C CC (SEQ ID NO 10) can be derived from the amino acid sequence F Y D V D P. Such an oligonucleotide primer could be used in PCR in combination with the primer A A G/A AA G/A CA XGC T/G/A AT (SEQ ID NO 11), derived from the bottom strand of the sequence that encodes I A C F F. (All sequences given 5′ to 3′; see Figure 3). X indicates A, G, C or T.

Preferably an oligonucleotide in accordance with

the invention, e.g. for use in nucleic acid amplification, has about 10 or fewer codons (e.g. 6, 7 or 8), i.e. is about 30 or fewer nucleotides in length (e.g. 18, 21 or 24).

Assessment of whether or not such a PCR product corresponds to resistance genes may be conducted in various ways. A PCR band from such a reaction might contain a complex mix of products. Individual products may be cloned and each one individually screened for linkage to known disease resistance genes that are segregating in progeny that showed a polymorphism for this probe. Alternatively, the PCR product may be treated in a way that enables one to display the polymorphism on a denaturing polyacrylamide gel and specific bands that are linked to the resistance gene may be preselected prior to cloning. Once a candidate PCR band has been cloned and shown to be linked to a known resistance gene, it may then be used to isolate cDNA clones which may be inspected for other features and homologies to either *RPP5*, *N* or *L6*. It may subsequently be analysed by transformation to assess its function on introduction into a disease sensitive variety of the plant of interest. Alternatively, the PCR band or sequences derived by analysing it may be used to assist plant breeders in monitoring the segregation of a useful resistance gene.

A further method of using the *RPP5* sequence to identify other resistance genes is to use computer

searches of expressed sequence tag (EST) and other DNA sequence databases to identify genes in other species that encode proteins with significant *RPP5* homology. For example, a homology score of at least 60 using one of the BLAST algorithms (Altschul *et al*, 1990) would indicate a candidate resistance gene.

Having obtained nucleic acid using any of these approaches, a nucleic acid molecule comprising all or part of the sequence of the obtained nucleic acid may be used in the production of a transgenic plant, for example in order to confer pathogen resistance on the plant.

Modifications to the above aspects and embodiments and further aspects and embodiments of the present invention will be apparent to those skilled in the art. All documents cited are incorporated herein by reference.

Figure 1 shows the genomic DNA sequence of the *RPP5* gene (SEQ ID NO. 1). Introns are shown in this Figure in non-capitalised letters. Features: Nucleic acid sequence - Translation start at nucleotide 1966; translation stop at nucleotide 6512.

Figure 2 shows predicted *RPP5* protein amino acid sequence (SEQ ID NO 2).

Figure 3 shows a comparison of the predicted amino acid sequence of the *RPP5* (SEQ ID NO 2), *N* (SEQ

ID NO 3) and *L6* (SEQ ID NO 4) genes. The protein sequences are aligned according to predicted protein domains. Figure 3 was produced using the PRETTYBOX and PileUp programs of the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package Version 7.2.

Figure 4 shows a contiguous nucleotide sequence (SEQ ID NO 5) encoding the amino acid sequence shown in Figure 2 (SEQ ID NO 2), and made by joining together the sequences of the exons of the sequence of Figure 1 (SEQ ID NO 1).

Cloning of the *Arabidopsis RPP5* gene

The *RPP5* gene was cloned using a map-based cloning strategy similar in principle to that used for the isolation of the tomato *Pto* gene, described briefly earlier.

(i) Assignment of RPP5 gene map locations

The map location of *RPP5* on the *Arabidopsis* RFLP map has been reported earlier (Parker *et al*, 1993). This paper describes in detail how two landraces of *Arabidopsis*, designated *Columbia* and *Landsberg erecta*, showed a differential response to a race of *Peronospora parasitica* designated *NoCo-2; Landsberg erecta* is resistant, and *Columbia* is sensitive. Recombinant inbred lines (Lister and Dean, 1993) had been constructed, derived from carrying out single

seed descent on *F2* seed derived from an *F1* between *Landsberg* and *Columbia*, and these recombinant inbred lines were tested for resistance or sensitivity to *NoCo-2*. This analysis showed that the *RPP5* gene lay on Chromosome 4 between the RFLP markers m226 and g4539. The DNA of *Landsberg* and *Columbia* was analysed using the RAPD (randomly amplified polymorphic DNA) technique (Williams *et al*, 1990) and polymorphisms between *Landsberg* and *Columbia* were analysed for linkage to *RPP5*. One polymorphism derived using the operon primer OPC18, which amplified a band in *Columbia* but not in *Landsberg* was absolutely linked to *RPP5*. This DNA band, of 540 bp (referred to as $OPC18_{540}$ in Parker *et al.*, 1993) was cloned and the resulting probe was designated the C18 probe.

(ii) Establishment of a physical map between marker m226 and marker g4539

The *Arabidopsis* genome project has as an objective the establishment of a physical map of Chromosome 4, and ultimately of the entire *Arabidopsis* genome. The C18 probe was used to identify hybridising yeast artificial chromosome (YAC) clones. This facilitated the establishment of a physical contig between 4539 and 226 incorporating other linked markers, such as g13683. The C18 RAPD band was cloned and used as a probe on *Columbia* and *Landsberg* genomic DNA. Hybridisation of this probe revealed a very

polymorphic small multi-gene family in these two genotypes. Hybridisation to recombinant inbred lines (Lister and Dean, 1993) showed that all members of this multi-gene family were absolutely linked to the resistance gene locus. Using the CAPS procedure (Konieczny and Ausubel, 1993) the individuals in an F2 population derived from selfing an F1 of a *Columbia* and *Landsberg* cross were screened for recombinants between the linked markers Ara-1 and 4539. The primers used for the Ara-1 locus were

Ara-1 5' TCG ACG ACT CTC AAG AAC CC 3'

Ara-2 5' CAC AAG CTA TAC GAT GCT CAC C 3'

This gave a 700 bp band in *Columbia* and *Landsberg* which, after digestion with Acc-1, cut *Landsberg* DNA giving a 360 bp and a 340 bp band. The primers used for the 4539 locus were

4539 F 5' GGT CAT CCG TTC CCA GGT AAA G 3'

4539 R 5' GGA CGT AGA ATC TGA GAG CTC 3'

After Hind III digestion, the *Columbia* 600 bp band remained uncut, whilst the *Landsberg* band was cut to give 480 bp and 120 bp fragments. In this way twenty-four additional recombinants were derived in this interval. Analysis of these recombinants showed that again all members of the C18 multi-gene family co-segregated exactly with *RPP5*. Since linked multi-gene families are a characteristic of disease resistance genes (Martin *et al*, 1993; Jones *et al*, 1994; Whitham

et al, 1994) we tested the hypothesis that the C18 band might hybridise to the *RPP5* gene. Cosmids were identified from a *Landsberg* binary vector cosmid library in the vector pCLD04541 (C. Dean, pers. com.; Bent *et al*, 1994) and cosmid clones that hybridised to the C18 probe were identified. Table 1 lists the hybridising clones. Each of these were used in transformation experiments with the readily transformable *Arabidopsis* landrace, No-0,which is sensitive to NoC0-2. A transformant was identified derived from transformation with cosmid 29L17, and self-progeny of this transformant segregated for resistance to *P. parasitica* NoC0-2. This demonstrated that the clone 29L17, which carries a band that hybridises to the C18 RAPD probe, carries a functional *Peronospora parasitica* resistance gene.

(iii) DNA sequence analysis of the 29L17 plant DNA insert

Cosmid DNA was prepared from 29L17, sonicated and cloned into pUC18 vector and randomly sequenced. Two hundred and forty (240) DNA sequencing reactions were performed on random clones that were identified as clones that hybridised to 29L17 insert DNA, i.e. clones that carried inserts of plant DNA. From a computer analysis of this DNA sequence data, a DNA sequence contig could be established comprising 14.3 kb of DNA. This DNA sequence was inspected for the

presence of sequences that encoded leucine-rich repeats. One such region, nucleotide 3000 to nucleotide 6138 in SEQ ID NO. 1, was found.

(iv) Analysis of a DNA rearrangement associated with an RPP5 mutation

One criterion for establishing whether or not a characterised region of plant DNA corresponds to the gene of interest is to inspect whether mutations in the corresponding gene, caused by ionizing radiation, are associated with DNA rearrangements in the region of interest. Fast neutron mutagenised *Landsberg* seed were screened with *Peronospora parasitica* for mutants to disease sensitivity. Three mutations were found and analysed by Southern blots for perturbations or rearrangements in DNA corresponding to the gene, carrying leucine rich repeats. One mutant line, FNB387, showed an altered pattern of Southern blot hybridisation. More detailed analysis showed that the perturbation consists of an insertion of 270 bp of DNA in the C-terminus of the reading frame that carries leucine-rich repeats. Sequence analysis of this region showed that an insertion of 270 bp had arisen from the duplication of several LRRs within the gene carried on cosmid 29L17. This provides very strong evidence that the *RPP5* gene corresponds to the reading frame that carries leucine-rich repeats.

(v) Demonstration that a subclone of 29L17 contains RPP5

To confirm that the gene identified by mutagenesis is not only necessary but also sufficient to confer disease resistance, a subclone of 29L17 was constructed in binary vector SLJ7292. The subclone, designated pRPP5-1, contained a 6304 bp DNA fragment defined by a *BglII* restriction enzyme site 5' to the gene (nucleotide 668 in SEQ ID NO. 1) and a *PstI* restriction enzyme site 3' to the gene (nucleotide 6971 in SEQ ID NO. 1). pRPP5-1 was used to transform *Arabidopsis* ecotype Columbia and shown to confer disease resistance.

(vi) RT-PCR analysis of the RPP5 transcript

First strand cDNA was prepared from seedling leaf messenger RNA and PCR amplification from this cDNA was performed using intron flanking primers. The primers were: for intron 1, 5'-GAGTTCGCTCTATCATCTCC and 5'-TTATTGCATTCGAAACATCATTG; for introns 2 and 3, 5'-AAATTGATCGTGCAAAGTCC and 5'-AAGATTCGCATTCTTCAAGATT; for intron 4, 5'-GAAGATGGATTTGTATAATTCC and 5'-TCAAATTCGGGCATCCAGTG. For intron 5 a nested PCR strategy was employed, an aliquot of the products of the first amplification being used as the template for the second. The primers used were: for the first amplification, 5'-TGGTGACACTTCCTTCCTCG and 5'-CCAAACTTTTGCAGTTGTTG; for the second amplification

5'-TCTCAATGTGAGCGGCTGCAAGC and 5'-AACTTGAGCAACCACTGAGATCG. Cloned PCR products were sequenced using a combination of vector-specific and insert-specific primers. Intron sequences are shown in lower case in SEQ ID NO. 1 (Figure 1) between the exon sequences shown in upper case.

(vii) Comparison of the RPP5 gene sequence with the sequences of other resistance genes

Comparison of the *RPP5* sequence to the genes *N* and *L6* reveals very strong homologies throughout the *N*-terminal region. These regions are highlighted in Figure 3. They include regions involved in nucleotide binding, designated Kinase-1a, Kinase-2, Kinase-3a. Kinase-1a is often referred to as the P-loop. Also, regions N-terminal to the nucleotide binding domain show conspicuous homologies. Primers were designed particularly to the conserved regions carrying the amino acid sequence F Y D V D P (amino acids 104 to 109 of *RPP5)* and to amino acids I A C F F (437-441 of *RPP5)*. When degenerate oligonucleotide primers based on amino acid sequence were used in PCR reactions, both on *Arabidopsis* genomic DNA and on cDNA made from RNA of other species, products were observed of the size consistent with the potential to encode resistance genes.

These primers could alone, or in combination with

other primers encoding conserved and non-conserved regions of the identified resistance genes, be used to isolate other homologous gene sequences which could include previously uncharacterized resistance genes.

Table 1:

Binary vector cosmid clones hybridising to C18

Binary vector: 04541

Transformed into No-o

3D23

27E2

29L17

38G10

42P15

Subsequently identified:

45F8

18A10

56G2

Table 2:

% amino acid identity between RPP5 and N; RPP5 and L6

	N exon1	N exon 2	N exon 3	N exon 4+5
RPP5				
exon 1	55			
exon 2		36		
exon 3			26	
exon 4,5+6				26

	L6 exon 1	L6 exon 2	L6 exon 3	L6 exon 4
RPP5				
exon 1	37			
exon 2		30		
exon 3			17	
exon 4,5+6				26

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CLAIMS

1. A nucleic acid isolate encoding a pathogen resistance gene whose expression in a plant can cause activation of a defence response in the plant, comprising a sequence of nucleotides encoding a polypeptide comprising the sequence of amino acids shown in Figure 2

2. Nucleic acid according to claim 1 wherein said activation is upon contact of the plant with a pathogen or corresponding elicitor molecule.

3. Nucleic acid according to claim 1 wherein the sequence of nucleotides comprises an encoding sequence shown in Figure 1.

4. Nucleic acid according to claim 1 wherein the sequence of nucleotides comprises an allele, derivative or mutant, by way of addition, insertion, deletion or substitution of one or more nucleotides, of an encoding sequence shown in Figure 1.

5. Nucleic acid encoding a pathogen resistance gene whose expression in a plant can cause activation of a defence response in the plant, comprising a sequence of nucleotides encoding a polypeptide, the polypeptide comprising an amino acid sequence which comprises an allele, derivative or mutant, by way of addition,

insertion, deletion or substitution of one or more amino acids, of the amino acid sequence shown in Figure 2;

with the proviso that the encoded polypeptide has at least about 60% homology with the amino acid sequence shown in Figure 2.

6. Nucleic acid encoding a pathogen resistance gene whose expression in a plant can cause activation of a defence response in the plant, comprising a sequence of nucleotides encoding a polypeptide, the polypeptide comprising an amino acid sequence which comprises an allele, derivative or mutant, by way of addition, insertion, deletion or substitution of one or more amino acids, of the amino acid sequence shown in Figure 2;

with the proviso that expression of the nucleic acid can cause said activation of a defence response upon contact of the plant with an Oomycete fungus, such as *Peronospora parasitica*, or an extract thereof.

7. Nucleic acid according to claim 5 or claim 6 wherein said activation is upon contact of the plant with a pathogen or corresponding elicitor molecule.

8. Nucleic acid which is a vector comprising nucleic acid according to any one of claims 1 to 7.

9. Nucleic acid according to claim 8 further comprising regulatory sequences for expression of said polypeptide.

10. Use of nucleic acid according to any one of the precedings claims in production of a transgenic plant.

11. A host cell comprising nucleic acid according to any one of claims 1 to 9.

12. A host cell according to claim 11 which is microbial.

13. A host cell according to claim 11 which is a plant cell.

14. A plant or any part thereof comprising a cell according to claim 13.

15. Seed, selfed or hybrid progeny or a descendant or derivative or extract of a plant according to claim 14, or any part thereof.

16. A method which comprises introduction of nucleic acid according to any one of claims 1 to 9 into a host cell.

17. A method according to claim 16 wherein the host

cell is a plant or microbial cell.

18. A method of conferring pathogen resistance on a plant, comprising expression from nucleic acid according to any one of claims 1 to 9, within cells of the plant, following an earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof.

19. A method according to claim 18 wherein the nucleic acid encodes an amino acid sequence shown in Figure 2.

20. An oligonucleotide comprising a sequence encoding an amino acid sequence conserved between RPP5 of *Arabidopsis* and another pathogen resistance genes or comprising a sequence complementary to a nucleotide sequence encoding a said amino acid sequence.

21. An oligonucleotide according to claim 20 wherein the pathogen resistance gene is *N* of tobacco or *L6* of flax.

22. An oligonucleotide according to claim 21 comprising a nucleotide sequence encoding one of the amino acid sequences:

(i)

F Y D/M V D P; and

(ii)

I A C F F/L

or comprising a nucleotide sequence complementary to a said encoding sequence.

23. An oligonucleotide according to claim 22 comprising a sequence selected from:

(i)

T T C/T T A C/T G A C/T G T X G A T/C C C;

(ii)

A A G/A A A G/A C A X G C T/G/A A T; and

(iii)

a sequence complementary to (i) or (ii).

24. An oligonucleotide which comprises a sequence which is a variant or derivative, by way of addition, insertion, deletion or substitution of one or more nucleotides, of the sequence of an oligonucleotide according to any one of claims 20 to 23.

25. A method of obtaining nucleic acid comprising a pathogen resistance gene comprising hybridisation of an oligonucleotide according to any one of claims 20 to 24, or a nucleic acid molecule comprising a said oligonucleotide, to target nucleic acid.

26. A method according to claim 25 involving use of nucleic acid amplification.

27. A method according to claim 25 or claim 26 wherein the hybridisation is followed by identification of successful hybridisation and isolation of target nucleic acid.

28. A method wherein following the obtaining of nucleic acid using the method of any of claims 25 to 27 a nucleic acid molecule comprising all or part of the sequence of the obtained nucleic acid is used in the production of a transgenic plant.

29. A method according to claim 28 wherein said nucleic acid molecule is used to confer pathogen resistance on said plant.

Figure 1

```
   1  atgtgaccag cacaaggaac aacccttcag gtgaaaagag aagagagcct
  51  ttgccatgtc atggaagaaa agcgctttta gattttctgc aagagacaat
 101  gtctgttcca attccaaacc atgaagtatc atccaaagct cctacgtcta
 151  tgagaaaacg ggtagctgct cttccaggga aagctgagaa ggaacttctt
 201  tatctgaccc caatgccact gtgctctaac ggtcggcctg aagcaggtgc
 251  agttctggtg taatcattga cttttgtttt tgtttcagag tttaatttaa
 301  accttaagaa gagtttttct gactttacta ggggacgtaa ttgagaagaa
 351  agacggtcct tgctcaggaa ccaaaggctt ccgagctcca gaggtacatc
 401  aactgtaact atagctcact tctcttaata cgagttaaat atgaaaatct
 451  tgtgccttca gactaaagtt gtaggcctga gctactcatt agcacatctc
 501  tgatgggaaa gctaattctc tggggctgtt cttggctgaa ttggtttgtg
 551  caggtttgct tcagatcttt gcaccaagga cctaagatag acgtgtggtc
 601  tgcgggagtt actttgttat acctcataat gggaaggaca cctttcactg
 651  gtgaccctga acagttaaga tctgacttga atgagtacac catcactcgg
 701  ttccactcgt ttcttctttc ttgccttctt acaacgacct tgtatttccc
 751  aggaacataa aggacattgc acaactacga ggcagtgaag aattatggga
 801  agtagccaag ctgcacaacc gtgaatcctc tttccctaag gtaacatctc
 851  atcttaccac tcttctgtac tctccattca tttatgagac acatatactt
 901  gctctgttct ttttaccgcc agcttaaata tcacataaaa gggttttcat
 951  tgttagcgtt ttgtgttcta ttgcttctac tgcactccca ttacagaaca
1001  tatatatctt aataatgtaa attgatgaca aagtgattaa atagatgatc
1051  gtgagagatg aaatcaggta gagttttgtg ttgttgtttc aggaattata
1101  cgagtcaagg tacttgaagg ggatggagtt gagaaaatgg ggcgaacgca
1151  acacaaaaag cagagagttt ctagacgcaa ttccacggcc gcttcttgaa
1201  ctcgttgata gatgtttgat agttaacccg aggcgacgaa tcagcgcaga
1251  ggatgctctc aagcacgagt tcttctatcc agtacatgaa acccttagaa
1301  accaaatgct ccttaaacag cagcaaatgc aatcgcagcc tacagttgtt
1351  gctgacgcac taagcgaaac tttaaactaa ttatacaatt cttaaaaact
1401  aaaagagtaa tttagcaaac tagagagtta attttcactt tagcaaacta
1451  gagagttaat ttaatttagc gaactaatta tattttcact ttagtataca
1501  attcttagtg ttaatttagt attttcactt atattatttg aattaaaatc
1551  ctcataatcg atatacttat tctcctaatc catgtgcatg tatgtattgg
1601  gaaacaagac tttgatatta aacaatcata agtacattct tacgataaaa
1651  tgtcttgtac aaggacaact gacacccaca aaatatgtgt gtttcaaaat
1701  atctgtgtag aggaaacgaa tgtaagtttc tgtctaattg cctagaactt
1751  gaaatattat ttctgtcttg tacaaagact aagacttatc ataattaagt
1801  gacaaccaca aaaattcaat ctctaaaaat atctttgtat gtagtgtaaa
1851  aaagctttcg aggaaagtaa gacgaagttt ctcctctctt tctcacacta
1901  tgtcttgctg atttacttct cttaaaaatc ttcgtctctt ctctgagttc
1951  gctctatcat ctcccATGGC GGCTTCTTCT TCTTCTGGCA GACGGAGATA
2001  CGACGTTTTT CCAAGCTTCA GTGGGGTTGA TGTTCGCAAG ACGTTCCTCA
2051  GCCATCTTCT CAAGGCTCTC GACGGCAAAT CAATCAATAC ATTCATCGAT
2101  CATGGAATCG AGAGAAGCCG CACAATCGCC CCTGAGCTTA TATCGGCGAT
2151  TAGAGAAGCT AGGATCTCAA TCGTCATCTT CTCTAAGAAC TATGCTTCTT
2201  CAACGTGGTG CTTAAATGAA TTGGTTGAGA TCCACAAGTG CTTTAATGAT
2251  TTAGGTCAAA TGGTGATTCC AGTTTTCTAC GACGTTGATC CTTCGGAAGT
2301  TAGAAAACAG ACCGGCGAAT TTGGAAAGGT CTTTGAAAAG ACATGCGAGG
2351  TCAGCAAGGA CAAACAACCA GGGGATCAGA AACAAAGATG GGTGCAAGCT
2401  CTCACAGATA TAGCAAATAT AGCCGGAGAG GATCTTCTGA ACGGgtacgt
2451  tgttatgatt ccaatatatc tgcttgcgtt ttcaattgtc tcagaactat
2501  atttttgcat agacttcggt tcttctttta ggggtgcttc ttaattgaca
2551  aaattgactt ttgttattag GCCTAATGAA GCGCATATGG TTGAAAAGAT
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Figure 1 (continued)

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2601  ATCCAATGAT GTTTCGAATA AACTTATCAC TCGGTCAAAG TGTTTTGATG
2651  ACTTCGTCGG AATTGAAGCT CATATTGAGG CAATAAAATC AGTATTGTGC
2701  TTGGAATCCA AGGAAGCTAG AATGGTCGGG ATTTGGGGAC AGTCAGGGAT
2751  TGGTAAGAGT ACCATCGGAA GAGCTCTTTT CAGTCAACTC TCTAGCCAGT
2801  TCCACCATCG CGCTTTCCTA ACTTATAAAA GCACCAGTGG TAGTGACGTC
2851  TCTGGCATGA AGTTGAGTTG GCAAAAAGAG CTTCTCTCGG AAATCTTAGG
2901  TCAAAAGGAC ATAAAGATAG AGCATTTTGG TGTGGTGGAG CAAAGGTTAA
2951  ATCACAAGAA AGTTCTTATC CTTCTTGATG ATGTGGATAA TCTAGAGTTT
3001  CTTAAGACCT TGGTGGGAAA AGCTGAATGG TTTGGATCTG GAAGCAGAAT
3051  AATTGTGATC ACTCAAGATA GGCAACTTCT CAAGGCTCAT GAGATTGACC
3101  TTGTATATGA GGTGAAGCTG CCATCTCAAG GTCTTGCTCT TAAGATGATA
3151  TCCCAATATG CTTTTGGGAA AGACTCTCCA CCTGATGATT TTAAGGAACT
3201  AGCATTTGAA GTTGCCGAGC TTGTCGGTAG TCTTCCTTTG GGTCTCAGTG
3251  TCTTGGGTTC ATCTTTAAAA GGAAGGGACA AAGATGAGTG GGTGAAGATG
3301  ATGCCTAGGC TTCGAAATGA TTCAGATGAT AAAATTGAGG AAACACTAAG
3351  AGTCGGCTAC GATAGGTTAA ATAAAAAAAA TAGAGAGTTA TTTAAGTGCA
3401  TTGCATGTTT TTTCAATGGT TTTAAAGTCA GTAACGTCAA AGAATTACTT
3451  GAAGATGATG TTGGGCTTAC AATGTTGGCT GAGAAGTCCC TCATACGTAT
3501  TACACCGGGT GGATATATAG AGATGCACAA TTTGCTAGAG AAATTGGGTA
3551  GAGAAATTGA TCGTGCAAAG TCCAAGGGTA ATCCTGGAAA ACGTCAATTT
3601  CTGACGAATT TTGAGGATAT TCGAGAAGTA TTGACCGAGA AAACTgtaag
3651  tttttcgcat ctccttaaac gttgtaatgc atgactttat atcaatataa
3701  tcgtaatttg gggattgata aacttaagca attgttgccc catgcgtaat
3751  taaaacgtag ctttgatgtg tcagaaaaat aaaaagggtt gcgattgtta
3801  agattatatt agttttcttc ggatttttt tcagGGGACC GAAACTCTTC
3851  TTGGAATACG TTTGCCACAC CCGGGATATC TTACGACAAG GTCGTTCTTA
3901  ATAGATGAAA AATCATTCAA AGGCATGCGT AATCTCCAAT ATCTAGAAAT
3951  TGGTTATTGG TCAGATGGGG TTCTACCTCA GAGCCTCGTT TATTTCCCTC
4001  GTAAACTCAA AAGGCTATGG TGGGATAATT GTCCATTGAA GCGTTTGCCT
4051  TCTAATTTTA AGGCTGAGTA TCTGGTTGAA CTCAGAATGG TGAATAGTAA
4101  GCTTGAGAAG CTGTGGGATG GAACTCAGgt actaattttt ttagtgatca
4151  atttctaaac ataaaaacta aaaataaaaa tgtttaaaat gttcattaac
4201  gtgtgtgctc tcttttcccc tattttgttt tcagCCCCTT GGAAGTCTCA
4251  AGAAGATGGA TTTGTATAAT TCCTACAAAT TGAAAGAAAT TCCAGATCTT
4301  TCTTTAGCCA TAAACCTCGA GGAATTAAAT CTTGAAGAAT GCGAATCTTT
4351  GGAGACACTT CCTTCCTCGA TTCAGAATGC CATTAAACTG AGGGAGTTAA
4401  ATTGTTGGGG GGGGCTATTA ATAGATTTAA AATCATTAGA AGGCATGTGT
4451  AATCTCGAAT ATCTATCAGT TCCTAGTTGG TCAAGTAGGG AATGCACTCA
4501  GGGCATCGTT TATTTCCCTC GTAAACTCAA AAGTGTATTG TGGACTAATT
4551  GTCCATTGAA GCGTTTGCCT TCTAATTTTA AGGCTGAGTA TCTGGTTGAA
4601  CTCATAATGG AGTACAGTGA GCTTGAGAAG CTGTGGGATG GTACTCAGgt
4651  actaattcta ttagtgataa taaatatgtt agaaaaacta aaaataaaaa
4701  tgtttaaaat gttcattaac gtgtgtgctc tcttttcccc tattttgtta
4751  tcagTCACTT GGAAGTCTCA AGGAGATGAA TTTGAGGTAT TCCAACAATT
4801  TAAAAGAAAT TCCAGATCTT TCTTTAGCCA TAAACCTCGA GGAATTAGAT
4851  CTTTTTGGAT GCGTATCTTT GGTGACACTT CCTTCCTCGA TTCAGAATGC
4901  CACTAAACTG ATCTATTTAG ATATGAGTGA ATGCGAAAAT CTAGAGAGTT
4951  TTCCAACCGT TTTCAACTTG AAATCTCTCG AGTACCTCGA TCTCACTGGA
5001  TGCCCGAATT TGAGAAATTT CCCAGCAATC AAAATGGGAT GTGCCTGGAC
5051  TAGATTATCT CGAACAAGAT TGTTTCCGGA AGGGAGAAAT GAGATCGTGG
5101  TAGAAGATTG TTTCTGGAAC AAGAATCTCC CTGCTGGACT AGATTATCTC
5151  GACTGCCTTA TGAGATGTAT GCCTTGTGAA TTTCGCTCAG AACAACTCAC
```

Figure 1 (continued)

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5201  TTTTCTCAAT GTGAGCGGCT GCAAGCTTGA GAAGCTATGG GAAGGCATCC
5251  AGgtacattg ttaatgctat gctgattttt gtttaccttc tgttatataa
5301  ctaattaagt atacccaaat ttgtttttat ggcttgtggt cgatccacgg
5351  ttatgtctta catacataca taataatgtt taattataat tttaaacata
5401  tataggtata aaattaaaat gattatcatc gataatgatt gaagcatacc
5451  aatgtttttt tcagTCGCTT GGAAGTCTCG AAGAGATGGA TCTGTCAGAA
5501  TCTGAAAACC TGAAAGAACT TCCAGATCTT TCAAAGGCCA CCAATCTGAA
5551  GCTTTTATGT CTCAGCGGGT GCAAAAGTTT GGTGACACTT CCTTCTACAA
5601  TTGGGAATCT TCAAAATTTG AGACGTTTGT ACATGAACAG ATGCACAGGG
5651  CTGGAGGTTC TTCCGACCGA TGTCAACTTG TCATCTCTCG AAACCCTCGA
5701  TCTCAGTGGT TGCTCAAGTT TGAGAACTTT TCCTCTGATT TCAACTAATA
5751  TTGTATGTCT CTATCTGGAA AACACCGCCA TTGAAGAAAT TCCAGATCTT
5801  TCAAAGGCCA CCAAGCTCGA GTCTTTGATA CTCAACAACT GCAAAAGTTT
5851  GGTGACACTT CCTTCTACAA TTGGGAATCT TCAAAATTTG AGACGTTTGT
5901  ACATGAACAG ATGCACAGGG CTGGAGCTTC TTCCGACCGA TGTCAACTTG
5951  TCATCTCTCG AAACCCTCGA TCTCAGTGGT TGCTCAAGTT TGAGAACTTT
6001  TCCTCTGATT TCAACTAGAA TCGAATGTCT CTATCTAGAA AACACCGCCA
6051  TTGAAGAAGT TCCCTGCTGC ATTGAGGATT TCACGAGGCT CACTGTACTA
6101  CGGATGTATT GTTGCCAGAG GTTGAAAAAC ATCTCCCCAA ACATTTTCAG
6151  ACTGACTAGT CTTACGCTCG CCGACTTTAC AGACTGTAGA GGTGTCATCA
6201  AGGCGTTGAG TGATGCAACT GTGGTAGCGA CAATGGAAGA TCACGTTTCT
6251  TGTGTACCAT TATCTGAAAA CATTGAATAT ACATGTGAAC GTTTCTGGGA
6301  TGCGTGTTCT GATTATTACT CTGATGACTT TGAGGTAAAT CGGAACCCAA
6351  TTAGATTGTC AACGATGACT GTCAACGATG TGGAGTTTAA GTTTTGTTGC
6401  TCCATTACGA TCAAAGAATG CGGTGTACGA CTCTTGTATG TCTATCAAGA
6451  AACAGAGCAC AACCAACAAA CTACGAGAAG CAAGAAGCGG ATGCGGGTAA
6501  GCCTTTTGCC Ataattagag ctgaaacttg taaagcaatc ttttgacttg
6551  atttgtttta taggatcaaa ataccatagc gacagactat ttgatagaat
6601  cgatcgtttg atatataatg cagatgacat cggggacatc tgaagaagat
6651  atcaacttac cctatggcca aattgtagcg gacacaggat tggccgctct
6701  aaatacagag ctttcgttag ggcagggaga agcatcatca tcaacatctc
6751  tagaggggga agctttgtgt gttgatgatt acatgataaa tgaagaacaa
6801  gatgaacaaa tacctatctt gtatcctgtt tatggtaact gaagcatctt
6851  tatcattctg ttttgctctt ttttaggata acttgggatc gaccattatt
6901  ataaatttat aatgataatg acaaaacgat ttcataggtt ttgacttttg
6951  acacaagcca ttttttctgc agatatagac gatgatatgt ggagatcatt
```

Figure 2.

```
   1  MAASSSSGRR RYDVFPSFSG VDVRKTFLSH LLKALDGKSI NTFIDHGIER
  51  SRTIAPELIS AIREARISIV IFSKNYASST WCLNELVEIH KCFNDLGQMV
 101  IPVFYDVDPS EVRKQTGEFG KVFEKTCEVS KDKQPGDQKQ RWVQALTDIA
 151  NIAGEDLLNG PNEAHMVEKI SNDVSNKLIT RSKCFDDFVG IEAHIEAIKS
 201  VLCLESKEAR MVGIWGQSGI GKSTIGRALF SQLSSQFHHR AFLTYKSTSG
 251  SDVSGMKLSW QKELLSEILG QKDIKIEHFG VVEQRLNHKK VLILLDDVDN
 301  LEFLKTLVGK AEWFGSGSRI IVITQDRQLL KAHEIDLVYE VKLPSQGLAL
 351  KMISQYAFGK DSPPDDFKEL AFEVAELVGS LPLGLSVLGS SLKGRDKDEW
 401  VKMMPRLRND SDDKIEETLR VGYDRLNKKN RELFKCIACF FNGFKVSNVK
 451  ELLEDDVGLT MLAEKSLIRI TPGGYIEMHN LLEKLGREID RAKSKGNPGK
 501  RQFLTNFEDI REVLTEKTGT ETLLGIRLPH PGYLTTRSFL IDEKSFKGMR
 551  NLQYLEIGYW SDGVLPQSLV YFPRKLKRLW WDNCPLKRLP SNFKAEYLVE
 601  LRMVNSKLEK LWDGTQPLGS LKKMDLYNSY KLKEIPDLSL AINLEELNLE
 651  ECESLETLPS SIQNAIKLRE LNCWGGLLID LKSLEGMCNL EYLSVPSWSS
 701  RECTQGIVYF PRKLKSVLWT NCPLKRLPSN FKAEYLVELI MEYSELEKLW
 751  DGTQSLGSLK EMNLRYSNNL KEIPDLSLAI NLEELDLFGC VSLVTLPSSI
 801  QNATKLIYLD MSECENLESF PTVFNLKSLE YLDLTGCPNL RNFPAIKMGC
 851  AWTRLSRTRL FPEGRNEIVV EDCFWNKNLP AGLDYLDCLM RCMPCEFRSE
 901  QLTFLNVSGC KLEKLWEGIQ SLGSLEEMDL SESENLKELP DLSKATNLKL
 951  LCLSGCKSLV TLPSTIGNLQ NLRRLYMNRC TGLEVLPTDV NLSSLETLDL
1001  SGCSSLRTFP LISTNIVCLY LENTAIEEIP DLSKATKLES LILNNCKSLV
1051  TLPSTIGNLQ NLRRLYMNRC TGLELLPTDV NLSSLETLDL SGCSSLRTFP
1101  LISTRIECLY LENTAIEEVP CCIEDFTRLT VLRMYCCQRL KNISPNIFRL
1151  TSLTLADFTD CRGVIKALSD ATVVATMEDH VSCVPLSENI EYTCERFWDA
1201  CSDYYSDDFE VNRNPIRLST MTVNDVEFKF CCSITIKECG VRLLYVYQET
1251  EHNQQTTRSK KRMRVSLLP
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Figure 3

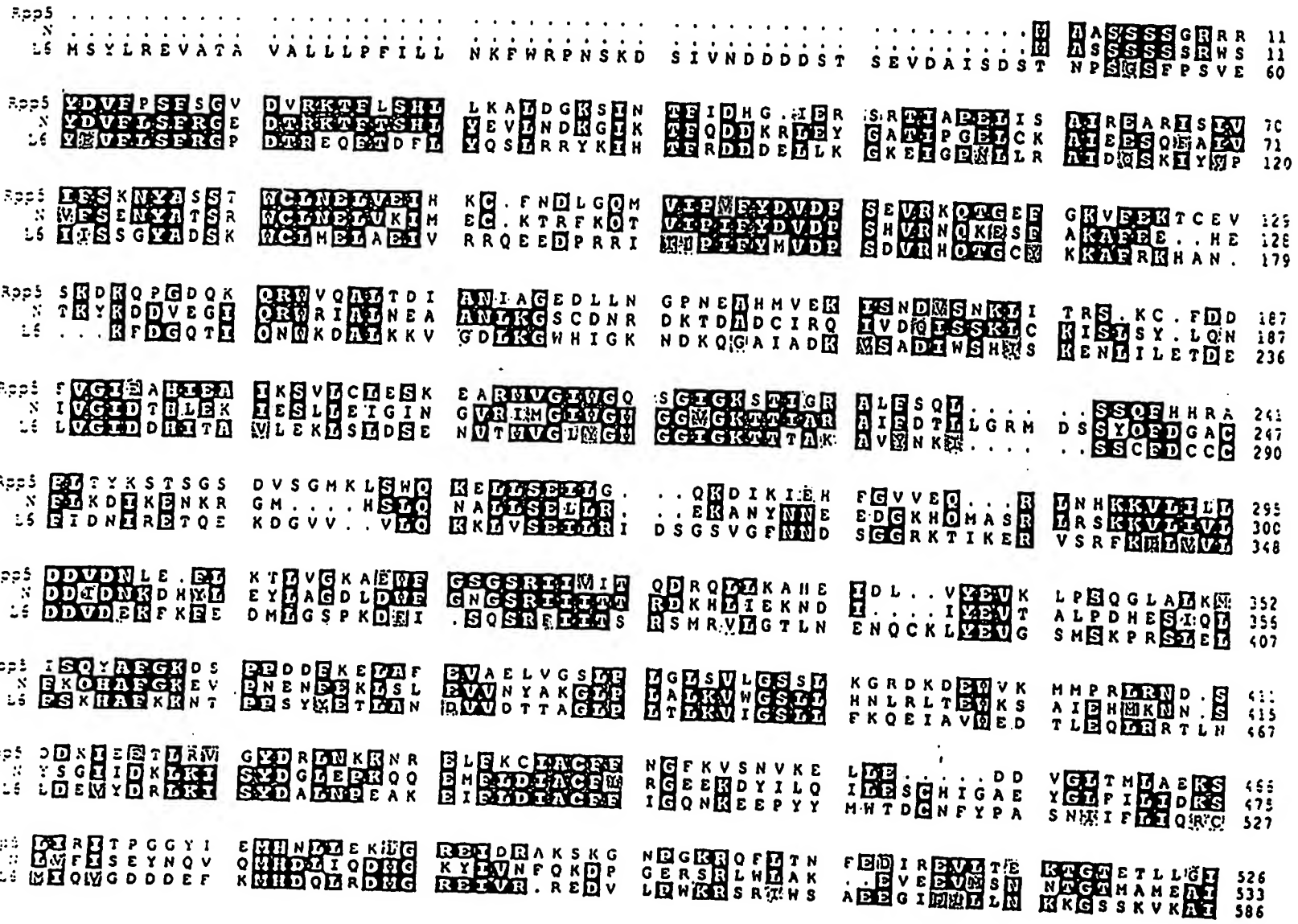

Figure 3 (continued)

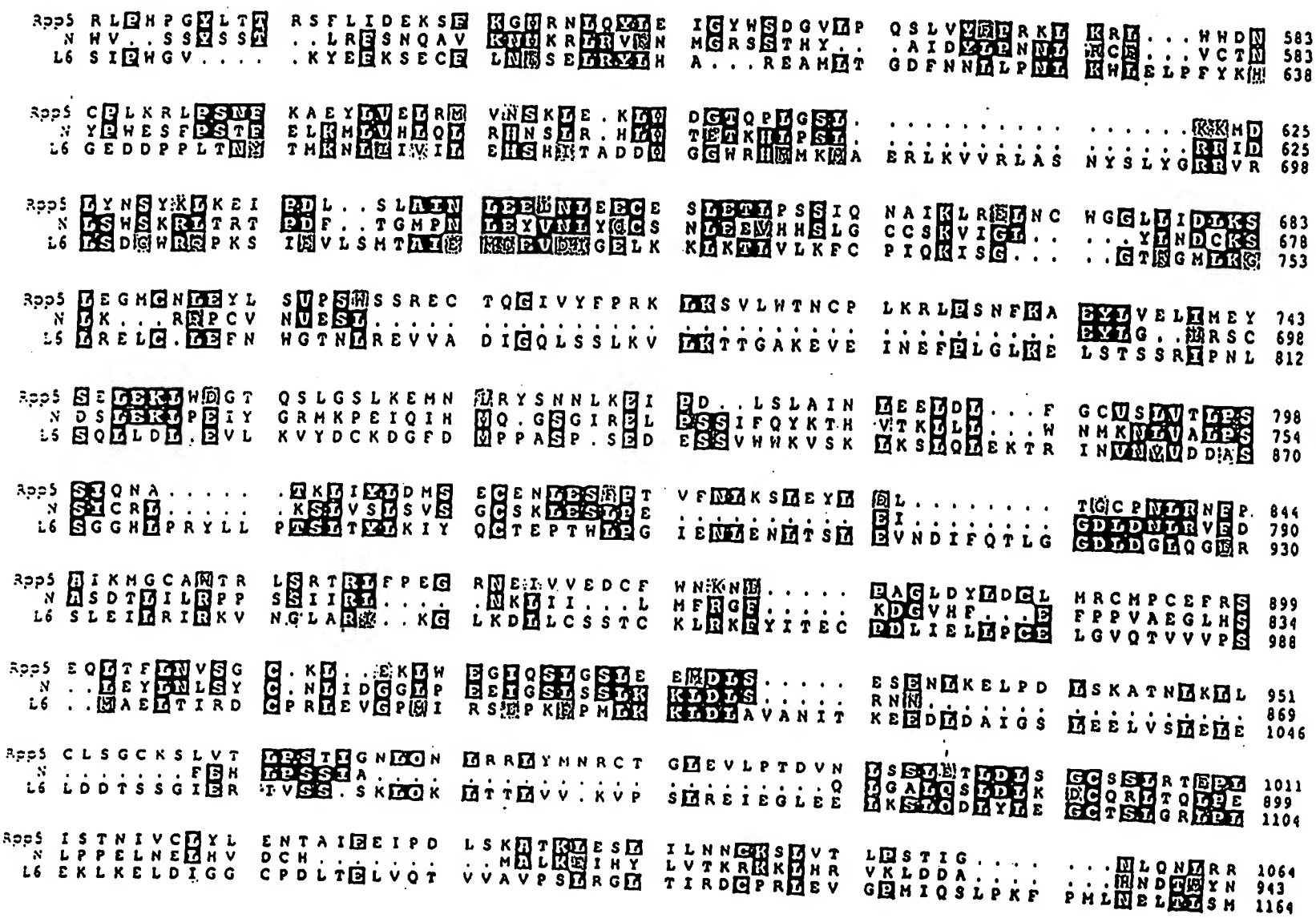

Figure 3 (continued)

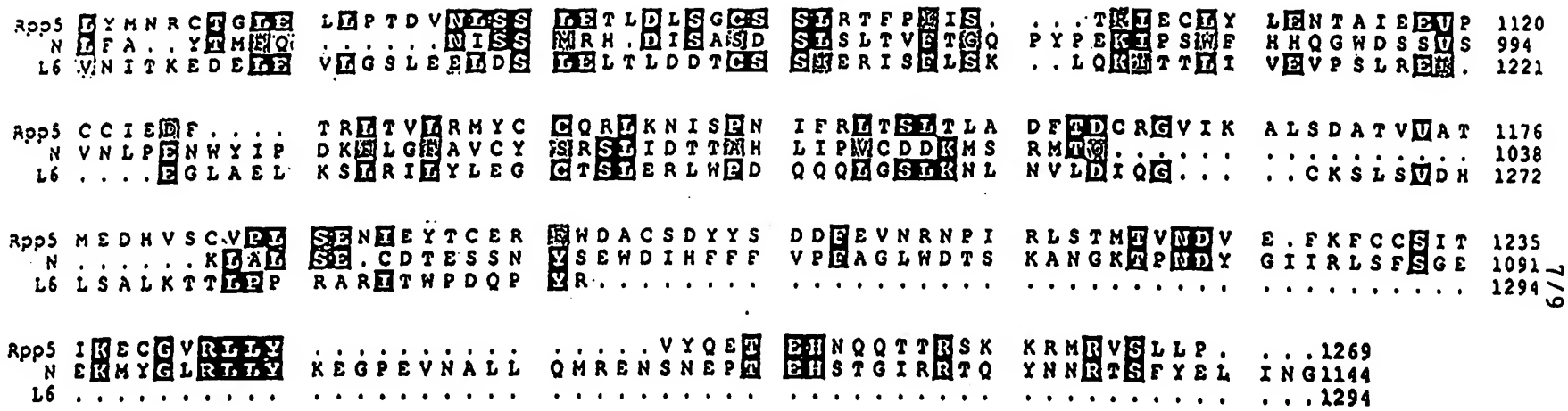

Figure 4

ATGGCGGCTT CTTCTTCTTC TGGCAGACGG AGATACGACG TTTTTCCAAG
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CTCTCGACGG CAAATCAATC AATACATTCA TCGATCATGG AATCGAGAGA
AGCCGCACAA TCGCCCCTGA GCTTATATCG GCGATTAGAG AAGCTAGGAT
CTCAATCGTC ATCTTCTCTA AGAACTATGC TTCTTCAACG TGGTGCTTAA
ATGAATTGGT TGAGATCCAC AAGTGCTTTA ATGATTTAGG TCAAATGGTG
ATTCCAGTTT TCTACGACGT TGATCCTTCG GAAGTTAGAA AACAGACCGG
CGAATTTGGA AAGGTCTTTG AAAAGACATG CGAGGTCAGC AAGGACAAAC
AACCAGGGGA TCAGAAACAA AGATGGGTGC AAGCTCTCAC AGATATAGCA
AATATAGCCG GAGAGGATCT TCTGAACGGG CCTAATGAAG CGCATATGGT
TGAAAAGATA TCCAATGATG TTTCGAATAA ACTTATCACT CGGTCAAAGT
GTTTTGATGA CTTCGTCGGA ATTGAAGCTC ATATTGAGGC AATAAAATCA
GTATTGTGCT TGGAATCCAA GGAAGCTAGA ATGGTCGGGA TTTGGGGACA
GTCAGGGATT GGTAAGAGTA CCATCGGAAG AGCTCTTTTC AGTCAACTCT
CTAGCCAGTT CCACCATCGC GCTTTCCTAA CTTATAAAAG CACCAGTGGT
AGTGACGTCT CTGGCATGAA GTTGAGTTGG CAAAAAGAGC TTCTCTCGGA
AATCTTAGGT CAAAAGGACA TAAAGATAGA GCATTTTGGT GTGGTGGAGC
AAAGGTTAAA TCACAAGAAA GTTCTTATCC TTCTTGATGA TGTGGATAAT
CTAGAGTTTC TTAAGACCTT GGTGGGAAAA GCTGAATGGT TTGGATCTGG
AAGCAGAATA ATTGTGATCA CTCAAGATAG GCAACTTCTC AAGGCTCATG
AGATTGACCT TGTATATGAG GTGAAGCTGC CATCTCAAGG TCTTGCTCTT
AAGATGATAT CCCAATATGC TTTTGGGAAA GACTCTCCAC CTGATGATTT
TAAGGAACTA GCATTTGAAG TTGCCGAGCT TGTCGGTAGT CTTCCTTTGG
GTCTCAGTGT CTTGGGTTCA TCTTTAAAAG GAAGGGACAA AGATGAGTGG
GTGAAGATGA TGCCTAGGCT TCGAAATGAT TCAGATGATA AAATTGAGGA
AACACTAAGA GTCGGCTACG ATAGGTTAAA TAAAAAAAAT AGAGAGTTAT
TTAAGTGCAT TGCATGTTTT TTCAATGGTT TTAAAGTCAG TAACGTCAAA
GAATTACTTG AAGATGATGT TGGGCTTACA ATGTTGGCTG AGAAGTCCCT
CATACGTATT ACACCGGGTG GATATATAGA GATGCACAAT TTGCTAGAGA
AATTGGGTAG AGAAATTGAT CGTGCAAAGT CCAAGGGTAA TCCTGGAAAA
CGTCAATTTC TGACGAATTT TGAGGATATT CGAGAAGTAT TGACCGAGAA
AACTGGGACC GAAACTCTTC TTGGAATACG TTTGCCACAC CCGGGATATC
TTACGACAAG GTCGTTCTTA ATAGATGAAA AATCATTCAA AGGCATGCGT
AATCTCCAAT ATCTAGAAAT TGGTTATTGG TCAGATGGGG TTCTACCTCA
GAGCCTCGTT TATTTCCCTC GTAAACTCAA AAGGCTATGG TGGGATAATT
GTCCATTGAA GCGTTTGCCT TCTAATTTTA AGGCTGAGTA TCTGGTTGAA
CTCAGAATGG TGAATAGTAA GCTTGAGAAG CTGTGGGATG GAACTCAGCC
CCTTGGAAGT CTCAAGAAGA TGGATTTGTA TAATTCCTAC AAATTGAAAG
AAATTCCAGA TCTTTCTTTA GCCATAAACC TCGAGGAATT AAATCTTGAA
GAATGCGAAT CTTTGGAGAC ACTTCCTTCC TCGATTCAGA ATGCCATTAA
ACTGAGGGAG TTAAATTGTT GGGGGGGGCT ATTAATAGAT TTAAAATCAT
TAGAAGGCAT GTGTAATCTC GAATATCTAT CAGTTCCTAG TTGGTCAAGT
AGGGAATGCA CTCAGGGCAT CGTTTATTTC CCTCGTAAAC TCAAAAGTGT
ATTGTGGACT AATTGTCCAT TGAAGCGTTT GCCTTCTAAT TTTAAGGCTG
AGTATCTGGT TGAACTCATA ATGGAGTACA GTGAGCTTGA GAAGCTGTGG
GATGGTACTC AGTCACTTGG AAGTCTCAAG GAGATGAATT TGAGGTATTC
CAACAATTTA AAAGAAATTC CAGATCTTTC TTTAGCCATA AACCTCGAGG
AATTAGATCT TTTTGGATGC GTATCTTTGG TGACACTTCC TTCCTCGATT
CAGAATGCCA CTAAACTGAT CTATTTAGAT ATGAGTGAAT GCGAAAATCT
AGAGAGTTTT CCAACCGTTT TCAACTTGAA ATCTCTCGAG TACCTCGATC

TCACTGGATG CCCGAATTTG AGAAATTTCC CAGCAATCAA AATGGGATGT
GCCTGGACTA GATTATCTCG AACAAGATTG TTTCCGGAAG GGAGAAATGA
GATCGTGGTA GAAGATTGTT TCTGGAACAA GAATCTCCCT GCTGGACTAG
ATTATCTCGA CTGCCTTATG AGATGTATGC CTTGTGAATT TCGCTCAGAA
CAACTCACTT TTCTCAATGT GAGCGGCTGC AAGCTTGAGA AGCTATGGGA
AGGCATCCAG TCGCTTGGAA GTCTCGAAGA GATGGATCTG TCAGAATCTG
AAAACCTGAA AGAACTTCCA GATCTTTCAA AGGCCACCAA TCTGAAGCTT
TTATGTCTCA GCGGGTGCAA AAGTTTGGTG ACACTTCCTT CTACAATTGG
GAATCTTCAA AATTTGAGAC GTTTGTACAT GAACAGATGC ACAGGGCTGG
AGGTTCTTCC GACCGATGTC AACTTGTCAT CTCTCGAAAC CCTCGATCTC
AGTGGTTGCT CAAGTTTGAG AACTTTTCCT CTGATTTCAA CTAATATTGT
ATGTCTCTAT CTGGAAAACA CCGCCATTGA AGAAATTCCA GATCTTTCAA
AGGCCACCAA GCTCGAGTCT TTGATACTCA ACAACTGCAA AAGTTTGGTG
ACACTTCCTT CTACAATTGG GAATCTTCAA AATTTGAGAC GTTTGTACAT
GAACAGATGC ACAGGGCTGG AGCTTCTTCC GACCGATGTC AACTTGTCAT
CTCTCGAAAC CCTCGATCTC AGTGGTTGCT CAAGTTTGAG AACTTTTCCT
CTGATTTCAA CTAGAATCGA ATGTCTCTAT CTAGAAAACA CCGCCATTGA
AGAAGTTCCC TGCTGCATTG AGGATTTCAC GAGGCTCACT GTACTACGGA
TGTATTGTTG CCAGAGGTTG AAAAACATCT CCCCAAACAT TTTCAGACTG
ACTAGTCTTA CGCTCGCCGA CTTTACAGAC TGTAGAGGTG TCATCAAGGC
GTTGAGTGAT GCAACTGTGG TAGCGACAAT GGAAGATCCG ATTTCTTGTG
TACCATTATC TGAAAACATT GAATATACAT GTGAACGTTT CTGGGATGCG
TGTTCTGATT ATTACTCTGA TGACTTTGAG GTAAATCGGA ACCCAATTAG
ATTGTCAACG ATGACTGTCA ACGATGTGGA GTTTAAGTTT TGTTGCTCCA
TTACGATCAA AGAATGCGGT GTACGACTCT TGTATGTCTA TCAAGAAACA
GAGCACAACC AACAAACTAC GAGAAGCAAG AAGCGGATGC GGGTAAGCCT
TTTGCCA

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/GB 96/00849

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 C12N15/82 A01N65/00 C12Q1/68 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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IPC 6 C12N A01N C12Q A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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2

Date of the actual completion of the international search	Date of mailing of the international search report
28 August 1996	03.09.96

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